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Applying Online Nano-UHPLC To Proteomics

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Overview

Online nano UHPLC allows longer, smaller ID columns improving chromatographic performance. Flow rate should be considered carefully.

Introduction

Ultra High Performance Liquid Chromatography (UHPLC) pushes the limits of feasible column designs through higher operational pressure. Migrating from nano-HPLC to nano-UHPLC and coupling directly to a mass spectrometer requires alterations to the setup to allow the increase in pressure. We systematically evaluate experimental parameters such as column dimensions and various stationary phases and how they apply to proteomic analysis.

Factors influencing the chromatographic part of Online LCMS performance include

- Column length
- Column inner diameter
- Flow rate
- Beads/solid phase material
- Bead size

Methods

An Easy nanoLC (Thermo, Odense, Denmark) modified to deliver up to 1000 bars was used with a LTQ or LTQ-Orbitrap XL (Thermo) for detection. A one column setup was used with predominantly in-house prepared columns having varying specifications: For baseline comparisons a 75 µm inner diameter (ID) 20 cm long column packed with 3 µm Dr. Maisch ReproSil-Pur C18-AQ beads was used. Alternative column dimensions (length and ID) were tested, as well as various flow-rates and different bead brands/sizes: HyperSil Gold (Thermo, San Jose, CA), Kinetex Core-Shell and 1.9 µm ReproSil-Pur.

Column preparation

All self-made columns were prepared as pulled emitters in-house using a Laser Puller (Sutter Instruments Model P-1800) and coated fused silica capillaries (e.g. Polymicro TSP075375). Pulled tips were inspected and packed with a self assembled frit (as in [1]). After the frit had formed under low pressure the column was packed in a bomb loader at 100 bars with methanol, overnight if necessary.

UHPLC Setup

HPLC grade solvents were prepared as A: 0.5% acetic acid in water (MilliQ) and B: 0.5% acetic acid, 80% acetonitrile in water. The solvents were degassed and exchanged regularly.

For acquisitions of Bovine Serum Albumin (BSA) a Thermo LTQ was used with a gradient from 13 to 33% B in 20 minutes, then to 100% B to wash and back to 2% for a certain amount of time depending on flow rate to allow all peptides to elute.

For complex samples a Thermo LTQ Orbitrap XL was used with a gradient going from 10 to 35% B in 120 minutes. The Orbitrap was set to acquire a full scan spectrum at resolution 60000, AGC target of 1e6 ions and a maximum injection time 500 ms. In parallel 10 CID MS^2 scans were acquired in the iontrap of the 10 most intense ions, dynamic exclusion was enabled with an exclusion period of 45 seconds.

Data processing

Data were processed using MaxQuant version 1.0.13.13 (see [4]) and searched against Uniprot Mouse (April 2011) using Matrix Science Mascot version 2.3. In-house developed software was used to extract features from BSA acquisitions in an automated manner.

Results

Initially a wide range of column dimensions and solid phase materials were tested looking for a golden solution yielding the highest number of identifications - with back-pressure as a non-limiting factor because of the UHPLC. A BSA digest was chosen as a simple mixture to narrow down the field early using short gradients on a lower-end mass spectrometer (primarily an old Thermo LTQ). A Total Ion Chromatogram (TIC) for BSA is depicted in Figure 1, with a few selected peptides of interest tagged.

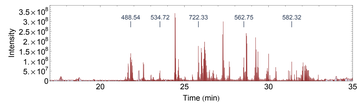

Figure 1: TIC of a few selected peptides from 50 fm BSA: m/z 488.54: TCVADESHAGCEK 3+, 534.72: QNCDQFEK 2+, 722.33: YICDNQDTISSK 2+, 562.75: ECCHGDLLECADDRADLAK 4+, 582.32: LVNELTEFAK 2+

Test varying the flow rate turned out to be of greater importance than expected initially and along with the potential increase in ionization efficiency it was included as a major factor in the considerations (reference [2] investigates this to more extreme values). Figure 2 illustrates the effect on a single BSA peptide. The signal intensity increases at lower flow rates while the peakwidth is affected but not in a uniform direction.

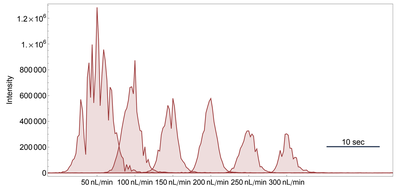

Figure 2: Extracted Ion Chromatograms for m/z 534.72 (QNCDQFEK, 2+) at flow rates from 50 to 300 nL/min on a 45cm long 50µm ID column.

To be able to capture the large quantity of information in a reproducible manner, a script was prepared to process and extract XICs from acquisitions using the MultiplierZ framework [4]. This script was setup to automatically process newly recorded BSAs producing up to 114 XICs per run. A post-process/viewer was written in Java, extracting centroids, median peak-widths (FWHM) and a few other pieces of information. Figure 3 shows median peak width from our standard 20cm long 75µm ID column versus a 45cm, ID 50µm high pressure column. Both columns are pulled columns packed to the tip with Reprosil-Pur 3µm beads, selected based on initial tests on several different materials and to reduce the number of variables. The optimal flow rate for both columns appeared to be lower than 250 nL/min but for standardization we decided to go for 250 nL/min for the short column and 125 nL/min for the long column in the next assessments tests.

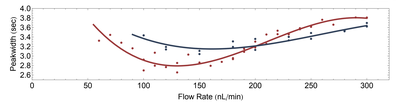

Figure 3: Flow rate versus observed peakwidth plots for two columns. 20cm, ID 75µm in blue, 45cm, ID 50µm in red.

Apart from the evidence in Figure 3, the column dimensions and flow rates were chosen for the following additional reasons: First, the internal volume of the two columns are identical thus having the roughly the same amount of beads, and second, the velocity of the liquid phase across the beads (in mm/sec) using half the flow rate on the 50µm column is similar: 0.24 versus 0.27 mm/sec (had the columns been empty).

Complex mixture

BSA digest is a wonderful substance to test column performance but the complexity is far to low to test the performance of modern MS equipment. Therefore we obtained a sample of mouse dendridic cells lysed and digested with trypsin, to get a representative of a sample used in proteomics studies. Proteins from roughly 50000 cells were used for each injection.

Figure 4 shows TIC from Orbitrap XL acquisitions of this complex sample on both the 20cm and 45cm column. The long but narrow column run at 125 nL/min versus 250 nL/min for the short. Two replicates were acquired on each column.

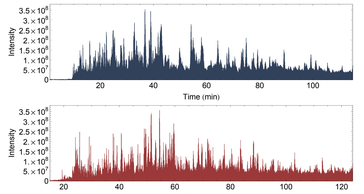

Figure 4: TIC for a complex sample on a short column (20cm, ID 75µm) in blue and long column (45cm, ID 50µm) in red. Retention time is shifted 15 minutes but otherwise highly reproducible (slope 1.0017, R^2 0.99).

MaxQuant was used to analyse the four acquisitions and 1072 proteins were identified with a false discovery rate less than 1% (both peptide and protein).
The number of identified peptides (distinct by sequence, Figure 5 top) was 2346 and 2506 for the short column and 3243 and 3272 for the long, representing an increase of 34% more peptides. Protein identifications went from 696 and 733 to 868 and 879, a 22% increase.

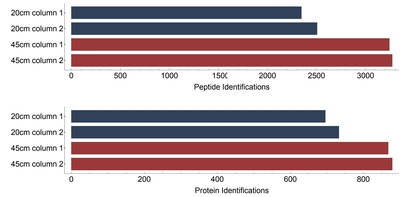

Figure 5: Comparison of distinct peptide identifications (top) and protein identifications (bottom) from mouse digest on two different columns, two replicates.

Both gradients were identical but due to the reduced flow rate the peptides eluted almost 15 minutes later on the long column. The long column had a significantly reduced number of peptides being sequenced more than once, but less than one third of the new identifications can be explained this way (based on the number of acquired spectra). Better ionization and/or better separation was observed in many cases. Figure 6 shows a nice example of peptides eluting from both columns.

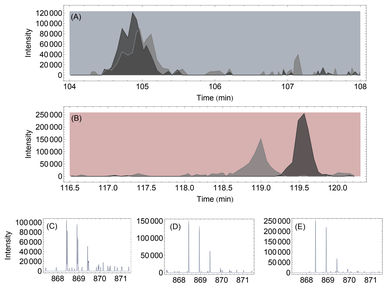

Figure 6: Comparing chromatographic separation for a short column (20cm, ID 75µm) and long column (45cm, ID 50µm). (A) XIC for two coeluting peptides on the short column (light-grey: m/z 868.46, dark-grey: m/z 868.42). (B) XIC on long column. (C) MS on peptides from short column. (D) and (E) separated peptides from long column.

Conclusion

- UHPLC broadens freedom of column selection and flow rates
- 50 µm columns proved superior
- Work in progress - the beauty of multivariate optimisation
- Systematic chromatographic assessment using low complexity analytes can be extrapolated to complex mixtures
- Flow rate optimisation cannot be neglected
- Ionization efficiency improves at lower flow rates
- Complex mixtures benefit from improved chromatography (!)
- 34% improvement achieved on a mouse cell lysate without acquisition method optimisation - which should be done in the future

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